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13. ABSTRACT (Maximum 200 words)

We are investigating the feasibility of using HER2/neu encoding genetic vaccines to elicit potent CD4<sup>+</sup> and CD8<sup>+</sup> T cell reactivity for the prevention and treatment of breast cancer. Two strategies of genetic vaccination, each with distinct advantages, will be assessed. The first strategy entails the use of alphavirus Venezuelan equine encephalitis virus (VEE)-replicons which selectively infect dendritic cells *in vivo*. Dendritic cells are characterized by an exceptional capacity to initiate and establish potent T cell immunity. Our second strategy is to employ a plasmid DNA (pDNA) vaccine encoding HER2/neu. pDNA vaccination has been shown to be effective in inducing persistent T cell responses in various model systems. VEE-replicon and pDNA vaccines encoding cytokines such as IL-12 and IL-18, will also be used to enhance anti-HER2/neu-specific CD4<sup>+</sup> Th1 cell and CD8<sup>+</sup> CTL activity.

To directly assess the efficacy of our approach to prevent tumor cell growth and/or eradicate established tumors, we will employ mice transgenic for a rat HER2/neu gene. These mice develop mammary tumors and pulmonary metastatic lesions. Furthermore, we will use mice transgenic for an HLA-A2.1 molecule to assess CD8<sup>+</sup> CTL reactivity to HER2/neu-specific peptide epitopes which have been proposed as targets for immunotherapy in a clinical setting.

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## The use of HER2/neu-specific genetic vaccines for the prevention and treatment of breast cancer.

### **INTRODUCTION.**

The long-term objective of this proposal is to establish a safe, effective, and persistent form of tumor antigen-specific immunotherapy for the prevention and treatment of breast cancer. Specifically, we are investigating the efficacy of tumor antigen encoding genetic vaccines to induce CD4<sup>+</sup> Th cell and CD8<sup>+</sup> CTL reactivity. We believe that recruitment of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells is necessary if not essential, to mediate potent and long-term immunity to tumor antigens, which typically are weak immunogens. Our model antigen is HER2/neu, an oncoprotein which is overexpressed in 30% of all breast and ovarian cancers. Two strategies of HER2/neu-specific genetic vaccination are being investigated, namely VEE-replicon- and pDNA-based vaccines. Each strategy will be assessed separately, however, we hypothesize that a combination of VEE-replicon and pDNA vaccination will prove to be the most effective form of immunotherapy necessary for prevention and eradication of established tumors. The rationale is to complement the strengths and weaknesses associated with each strategy. For example, administration of a VEE-replicon provides a unique approach to selectively infect dendritic cells *in vivo*, and in turn initiate potent T cell immunity. However, expression mediated by a VEE-replicon is only transient. On the other hand, the stability and long-term expression of an immunogen encoded by pDNA typically results in persistent T cell and antibody immunity. Yet, it is unlikely that the magnitude of the T cell response elicited via pDNA vaccination will be sufficient to eradicate established tumors. We predict that these two strategies together will elicit a robust and persistent T cell response. In a further attempt to enhance immunotherapeutic efficacy, VEE-replicon and pDNA vaccines encoding cytokines such as IL-12 and IL-18 known to promote CD4<sup>+</sup> Th1 and CD8<sup>+</sup> CTL reactivity, will be employed. To directly determine the immunotherapeutic efficacy of the treatment regimes, we have established a unique model system incorporating mice transgenic for HER2/neu and HLA-A2.1. In this way, we can: i) critically assess the effectiveness of targeting immunity to a self antigen, ii) directly determine the efficacy of our treatment regimes to prevent tumor progression and eradicate established tumors, and iii) analyze CD8<sup>+</sup> CTL reactivity to HER2/neu-specific epitopes which may have direct application for immunotherapy. In summary, this proposed work should provide the necessary insight to establish a rational, effective, and safe form of HER2/neu-specific genetic vaccination for the long-term prevention and treatment of breast cancer. In addition, this work will generate therapeutic reagents which if effective, can be directly applied to a clinical setting.

### **BODY.**

**Specific Aim 1. Determine the optimal conditions for pDNA and VEE-based replicon vaccination to induce HER2/neu-specific CD4<sup>+</sup> Th cell and CD8<sup>+</sup> CTL reactivity.**

**Task 1. *Characterization of HER2/neu encoding VEE-replicon recombinant.***

Initially, we had established pDNA and VEE-replicon recombinants encoding full length human HER2/neu. However, the FVB/neu transgenic mice which will be employed to assess the immunotherapeutic efficacy of genetic vaccination express rat HER2/neu. The homology between rat and human HER2/neu protein is approximately 90%. This difference in protein homology may be sufficient to have human HER2/neu recognized more so as a foreign antigen as opposed to a self protein in the FVB/neu mice. Since the success of

this approach and any other form of tumor antigen-specific immunotherapy is dependent on the degree of immunity achieved to a self protein (to which various levels of self tolerance may exist), we have generated a second panel of pDNA and VEE-replicon recombinants encoding full length rat HER2/neu. In this way, we can critically assess the immunotherapeutic efficacy of the respective genetic vaccines targeting a "bonafide" self antigen.

A 4.6 kilobase (kb) pair cDNA encoding rat HER2/neu was engineered to be flanked by either ClaI or HindIII restriction enzyme sites, in order to facilitate subcloning into the VEE-replicon (pVR2) or pDNA (Jw4303) constructs, respectively. To determine whether the VEE-replicon recombinant was functional, pVR2-neu DNA was linearized, and *in vitro* transcribed RNA was transfected into baby hamster kidney (BHK) cells. HER2/neu protein was detected via immunohistological staining in BHK cells transfected with pVR2-neu RNA, but not in mock transfected BHK cells (Figure 1). To determine whether the pDNA construct was functional, COS7 cells were transfected with pDNA-neu. Cell lysates were subsequently prepared, resolved by SDS-PAGE, and analyzed via Western blot. As demonstrated in Figure 2, HER2/neu protein migrating at approximately 185 kilodaltons was detected in pDNA-neu, but not in mock transfected COS7 cells. These results demonstrate that both VEE-replicon and pDNA vaccines encoding rat HER2/neu are functional.

We are currently generating constructs to express overlapping fragments of recombinant rat HER2/neu. Recombinant protein is required to detect HER2/neu-specific CD4<sup>+</sup> T cell reactivity. Specifically, the pTRCHIS *E.coli* expression system (Invitrogen) is being utilized to express histidine tagged HER2/neu fragments. Initially, pTRCHIS recombinants encoding full length, or a panel of four overlapping fragments spanning the entire length of the molecule were established. No protein expression, however, was detected from any of these recombinants. It is possible that the size of the respective recombinant proteins and/or other intrinsic properties precluded efficient expression in *E.coli*. With this in mind, a panel of eight pTRCHIS recombinants encoding smaller sized protein fragments is currently being established.

#### Task 2. Complete construction and testing of pDNA and VEE-replicon recombinants expressing IL-12 and IL-18.

We have completed and tested pDNA constructs encoding murine IL-12 (pDNA-IL12), murine IL-18 (pDNA-IL18) or both IL-12 and IL-18 (pDNA-IL12/18). Significant amounts of IL-12 were detected via ELISA in supernatants harvested from COS7 cells transfected with either pDNA-IL12 or pDNA-IL12/18 (Figure 3A). Furthermore, enhanced levels of IFN $\gamma$  secretion were detected in splenocyte cultures suboptimally stimulated with concanavalin A (ConA) and incubated with supernatant prepared from COS7 cells transfected with pDNA-IL18, or pDNA-IL12/18 (Figure 3B). Interestingly, a greater amount of IFN $\gamma$  secretion was induced with supernatant prepared from cells transfected with pDNA-IL12/18 versus pDNA-IL12 or pDNA-IL-18. This observation is consistent with previous reports demonstrating that together, IL-12 and IL-18 have a synergistic effect on induction of IFN $\gamma$  secretion (1). We have also generated VEE-replicons encoding IL-12 (pVR2-IL12) and IL-18 (pVR2-IL18). IL-12 was detected in supernatants prepared from BHK cells transfected with pVR2-IL12 RNA (Figure 3A), whereas enhanced IFN $\gamma$  secretion was observed in cultures incubated with supernatant from pVR2-IL18 RNA transfected BHK cells (Figure 3B). These results demonstrate that the pDNA-IL12, pDNA-IL18, pDNA-IL12/18, pVR2-IL12, and pVR2-IL18 recombinants are all functional. We have recently established a VEE-replicon encoding both IL-12 and IL-18, and are currently assessing the recombinant for protein expression.

**Task 3. Characterize and optimize CD4<sup>+</sup> Th cell and CD8<sup>+</sup> CTL reactivity in A2.1/K<sup>b</sup> FVB mice following treatment with HER2/neu- and cytokine-specific pDNA and VEE-replicon encoding recombinants.**

To determine the *in vivo* function of the pDNA-neu vaccine, FVB/n mice transgenic for the human HLA class I fusion molecule HLA-A2.1 (A2.1/K<sup>b</sup>) received three intramuscular injections of 50 µg of pDNA in each quadricep over a period of three weeks. Two weeks after the final injection, mice were sacrificed and HER2/neu-specific CD8<sup>+</sup> CTL reactivity measured. The target cells used in this assay were H-2<sup>a</sup> expressing 1T22 tumor cells which were infected with wild type vaccinia virus (VV-SC11) or a recombinant encoding rat HER2/neu (VV-neu). Uninfected 1T22 cells were used as an additional control. As demonstrated in Figure 4, a robust HER2/neu-specific CD8<sup>+</sup> CTL response was detected. These results clearly indicate that a CD8<sup>+</sup> CTL response can be induced by administration of pDNA-neu. Experiments are underway to determine whether CD8<sup>+</sup> CTL reactivity can be enhanced by co-immunization with pDNA-neu and the cytokine recombinants. In addition, we have ongoing experiments in which A2.1/K<sup>b</sup> mice have been immunized with VEE-neu or VEE-neu plus VEE-replicons encoding IL-12 and/or IL-18.

Recently, we demonstrated that significant CD8<sup>+</sup> CTL reactivity can be induced via immunization with a VEE-replicon encoding human papillomavirus (HPV) expressed E7. A2.1/K<sup>b</sup> transgenic mice received three foot pad injections of 2x10<sup>6</sup> VEE-E7 infectious units (IU) over 6 weeks. Three weeks subsequent to the final injection, CD8<sup>+</sup> CTL reactivity was assessed. Figure 5 demonstrates that EL4 target cells expressing A2.1/K<sup>b</sup> and infected with a vaccinia recombinant encoding HPV E7 (VV-E7), but not VV-SC11 were specifically lysed.

**Specific Aim 2. Determine the efficacy of HER2/neu-specific pDNA and VEE-replicon vaccination to prevent and eradicate breast tumor cell growth in HER2/neu transgenic mice.**

**Task 1. Determine efficacy of VEE-replicon and pDNA administration to prevent tumor progression in FVB/neu x A2.1/K<sup>b</sup> mice.**

An important issue for this study is the immune status of the rat HER2/neu protein in FVB/neu (or FVB/neu x A2.1/K<sup>b</sup>) mice. For example, eliciting a specific T cell response in FVB/neu mice may prove to be highly problematic if the animals are highly tolerant to HER2/neu. To investigate this possibility further, A2.1/K<sup>b</sup> versus FVB/neu x A2.1/K<sup>b</sup> mice were immunized subcutaneously three times with 100 µg of the HER2/neu derived peptide E75 prepared in incomplete Freund's adjuvant (IFA). The E75 peptide, which is HLA-A2.1 restricted and conserved between human and rat, has previously been shown to be recognized by CD8<sup>+</sup> CTL infiltrating ovarian tumors in patients (2). As demonstrated in Figure 6, CD8<sup>+</sup> CTL reactivity specific for E75 peptide was detected in FVB/neu x A2.1/K<sup>b</sup> mice, and this response was similar to that observed in A2.1/K<sup>b</sup> mice immunized with E75. These results suggest that self tolerance to HER2/neu is incomplete in the FVB/neu mice.

**Task 2. Determine efficacy of VEE-replicon and pDNA administration to eradicate established tumor foci in the mammary glands and pulmonary metastatic lesions in FVB/neu x A2.1/K<sup>b</sup> mice.**

Once the optimal parameters for immunization with the VEE-replicon and pDNA vaccines have been determined in Specific Aim 1, we will proceed with Tasks 1 and 2 for Specific Aim 2. We anticipate that this work will be initiated approximately half way through the second year of funding.

### **KEY RESEARCH ACCOMPLISHMENTS.**

- Establishment of functional VEE-replicon and pDNA vaccines encoding rat HER2/neu.
- Establishment of functional VEE-replicon and pDNA vaccines encoding murine IL-12, IL-18, or both.
- Demonstration that immunization with pDNA-neu elicits significant HER2/neu-specific CD8<sup>+</sup> CTL reactivity in A2.1/K<sup>b</sup> mice.
- Demonstration that immunization with an oncoprotein encoding VEE-replicon elicits CD8<sup>+</sup> CTL reactivity.

### **REPORTABLE OUTCOMES.**

Not applicable.

### **CONCLUSIONS.**

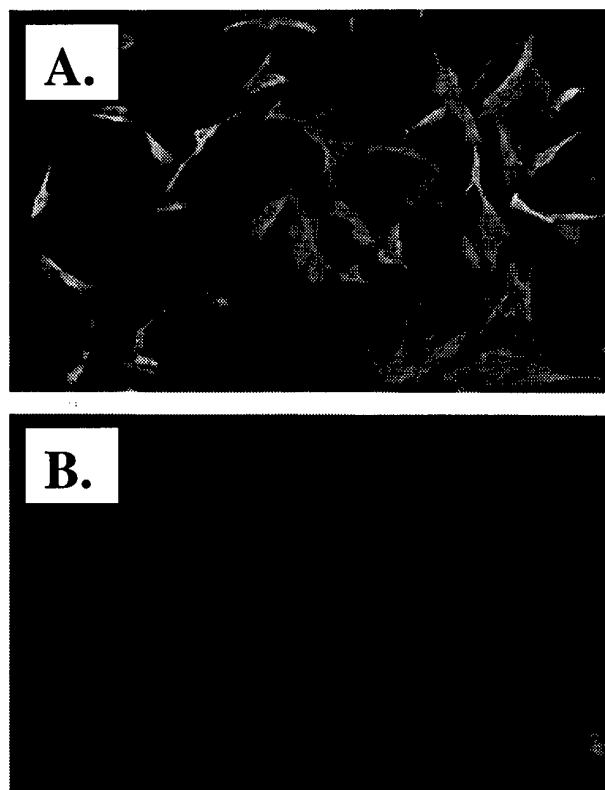
To date, we have generated and tested essentially all of the VEE-replicon and pDNA recombinants that will be required for the remainder of this study. Importantly, we have evidence demonstrating that significant CD8<sup>+</sup> CTL responses can be induced by either pDNA or VEE-replicon immunization.

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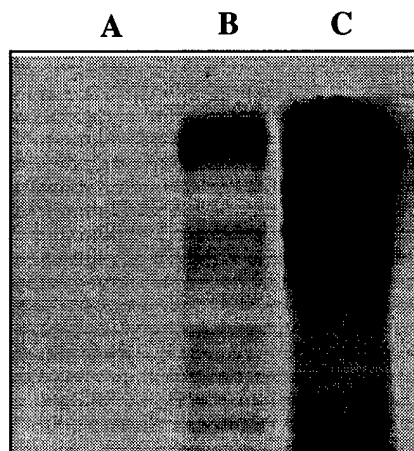
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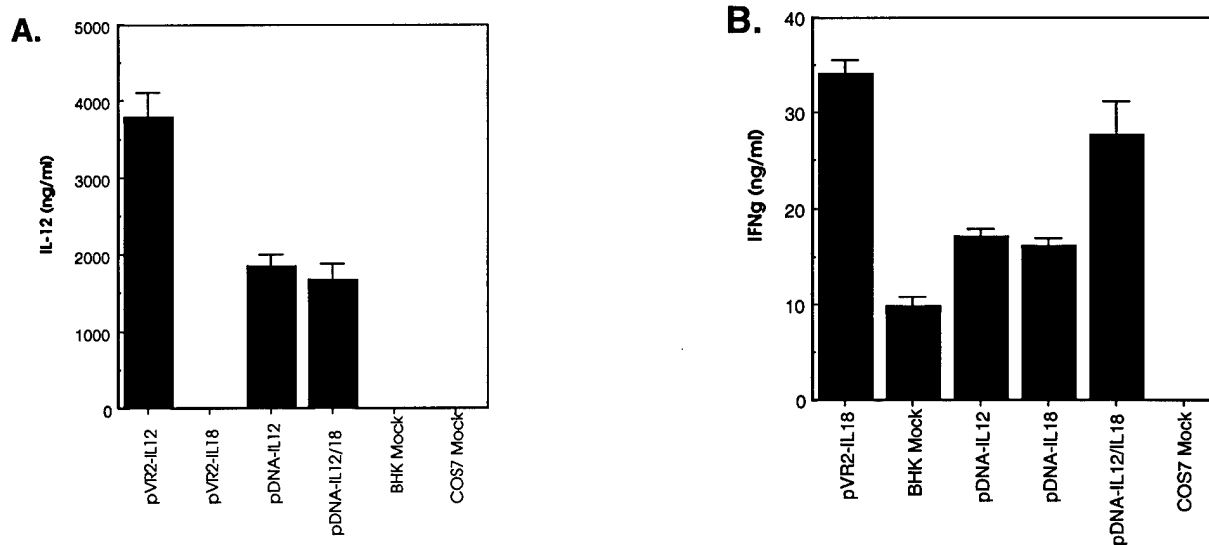
**APPENDICES.**



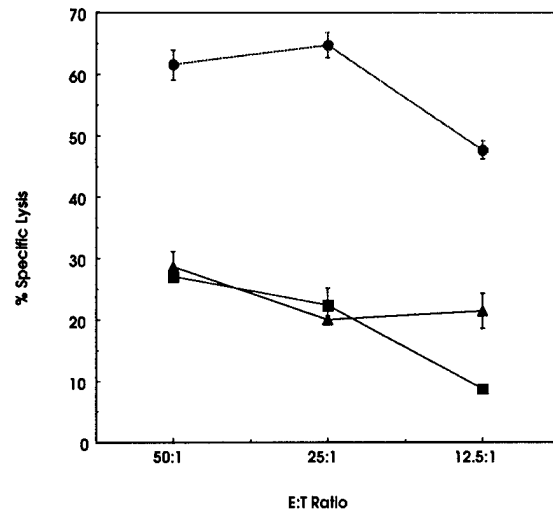
**Figure 1.** HER2/neu protein expression in BHK cells transfected with pVR2-neu RNA. BHK cells were transfected via electroporation with 20  $\mu$ g of pVR2-neu encoded RNA (A) or mock transfected (B), and plated on chamber slides for 20 hours. Cells were fixed and incubated with a polyclonal goat anti-rat neu antibody (Santa Cruz Biotechnology). Cells were then incubated with biotinylated anti-goat IgG, and finally with streptavidin-FITC.



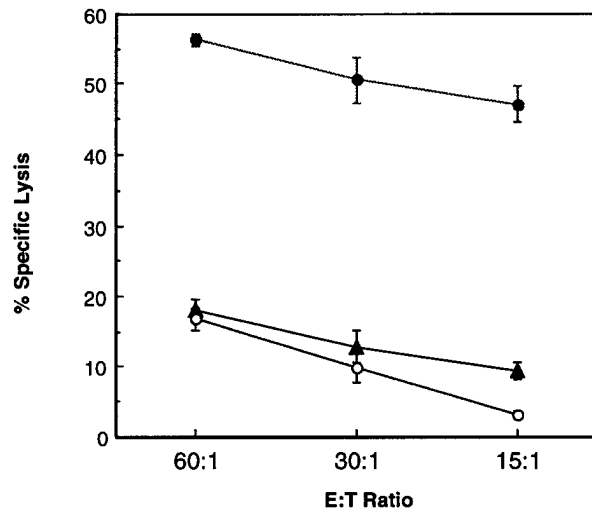
**Figure 2.** HER2/neu protein expression in COS7 cells transfected with pDNA-neu. Western blot analysis of cell lysates prepared from COS7 cells mock transfected (A), or transfected with 2.0 µg of pDNA-neu (B&C) via Lipofectamine (GIBCO-BRL). A goat anti-rat neu antibody (Santa Cruz Biotechnology) was used for detection.



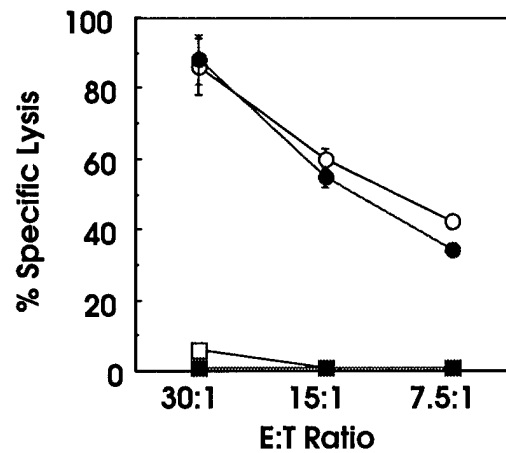
**Figure 3.** Protein expression by cytokine encoding pDNA and VEE-replicon recombinants. Supernatants harvested from cells transfected with pDNA or VEE-replicon RNA were tested via ELISA for IL-12 (A) or the capacity to enhance levels of IFN $\gamma$  secretion in splenocyte cultures suboptimally stimulated with Con A (B).



**Figure 4.** HER2/neu-specific CD8<sup>+</sup> CTL reactivity is elicited in FVB A2.1/K<sup>b</sup> mice immunized with pDNA-neu. FVB A2.1/K<sup>b</sup> mice were immunized intramuscularly with a total of 100 µg of pDNA-neu three times. <sup>51</sup>Cr labeled 1T22 target cells were infected with VV-SC11 (square), VV-rat neu (circle), or left untreated (triangle). CD8<sup>+</sup> CTL effector function was determined by varying the effector to target (E:T) cell ratio and measuring percent specific lysis via <sup>51</sup>Cr release.



**Figure 5.** Immunization with a VEE-replicon elicits CD8<sup>+</sup> CTL reactivity. A2.1/K<sup>b</sup> mice were immunized three times in the foot pad with VEE-replicon encoding HPV E7. <sup>51</sup>Cr labeled EL4-A2.1/K<sup>b</sup> target cells were infected with VV-SC11 (triangles), or the E7 encoding vaccinia virus VV-E7 (closed circles) or were left untreated (open circles).



**Figure 6.** CD8<sup>+</sup> CTL reactivity is similar in FVB/neu x A2.1/K<sup>b</sup> versus A2.1/K<sup>b</sup> mice immunized with the HER2/neu-specific E75 peptide. FVB/neu x A2.1/K<sup>b</sup> (closed symbols) or A2.1/K<sup>b</sup> mice (open symbols) were immunized three times with 100 µg E75 peptide in IFA. <sup>51</sup>Cr labeled T2 target cells were pulsed with E75 peptide (circle) or the HPV derived E7 peptide 86-93 (square), known to bind HLA-A2.1.